# New Use of Cyanosilane Coupling Agent for Direct Binding of Antibodies to Silica Supports. Physicochemical Characterization of Molecularly Bioengineered Layers

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A simple protocol to fix biological species to silica-based surfaces (silica microbeads and glass slides), using a bifunctional silane reagent (3-cyanopropyl dimethyl chlorosilane), is presented. This silane reagent was used without further derivatization. This system led to strong, but not covalent, linkage of antibodies through their glycosylated regions (OH groups) to solid supports. The use of a microsized sample revealed that the coupling process depends not only on physicochemical interactions but also on steric phenomena, and in this case, it was shown that a molecule acting as a spacer was required for more efficient cell fixation. Here, monoclonal mouse antibodies against the CD45 molecule expressed on rat lymphocytes (MAR anti CD45 Ab) were linked to lymphocytes, and as spacers, sheep anti-mouse antibodies (SAM Ab) were immobilized on silica surfaces, allowing the cells to stick to the floating hollow silica microbeads by simple incubation. Under such conditions, a single microbead can fix several cells. The potential of this hollow, low-density support is in ultrasound applications, for the destruction by cavitation phenomena of cells selectively fixed onto such a support. Such a study can serve as a basic model for various microbiosystems involving cell manipulation.

## INTRODUCTION

Since the first work of Nelson and Griffin (*1*), fixing biologicals onto a variety of solid inorganic supports has become a widely used technique.

A great number of biomedical applications, especially diagnostic, involve binding biospecies (antigen, antibody, and enzyme) to various solid matrixes. For example, biosensing applications require the biospecies involved to be grafted onto silicon, polymers, or metallic-based materials (*2*, *3*). For immunoaffinity chromatography, functionalized beads are required. The various functionalization methods are often tedious, rather expensive, and can denature the biological species (*4*). Moreover, the efficiency of the grafting processes must often be characterized through topographical studies (atomic force microscopy) or analytical investigations. For this purpose, substrates such as mica or glass slides with similar reactivity and with a well-controlled surface state (low roughness) are used (*5*, *6*).

Various chemical reagents can be used to modify surfaces in order to immobilize proteins (*7*). For example, surfaces can be activated with reagents such as BrCN or derivatized with linkers such as triazines, epoxy, aryl azides, isothiocyanates, *N*-hydroxysuccinimides, silanes, etc. Bifunctional silanes with a $(MeO)_3Si(CH_2)_nX$ structure, where X represents a reactive moieties such as an amine or a glycidoxy group, were used extensively in the 1970s as coupling agents to link proteins to various inorganic solid supports (*8*). Very often, after silanization, the reactive moieties must be derivatized prior to protein immobilization. This additional step is not always easy to realize and a direct coupling procedure without the use of an intermediary step would be of interest.

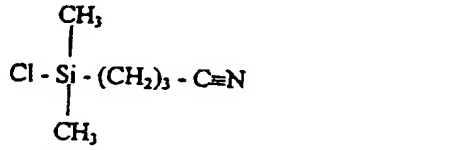


**Figure 1.** Bifunctional silane reagent: 3-cyanopropyl dimethyl chlorosilane.

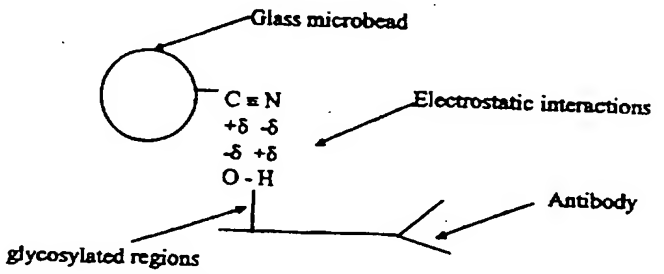


**Figure 2.** Antibody coupling to silica or glass surfaces.

In this paper, a simple protocol to fix biological species to silica-based surfaces by using a bifunctional silane reagent (3-cyanopropyl dimethyl chlorosilane, cf. Figure 1) is presented. This silane reagent was used without further derivatization. Such a system has already been shown to induce a strong, but not covalent linkage, of antibodies to solid supports, through their glycosylated regions (OH groups) (cf. Figure 2) (*9*). Moreover, antibodies fixed onto a cyanosilanized silica substrate have been

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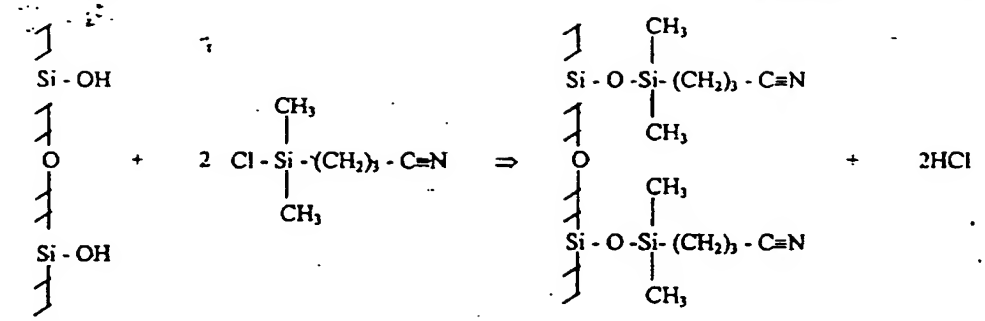



**Figure 3.** Activation of glass surface by silane reagent.

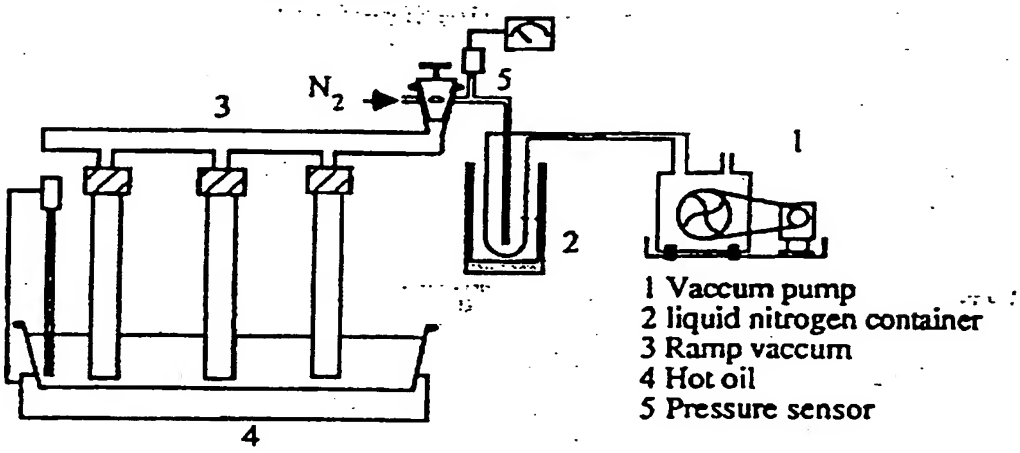



**Figure 4.** Vapor phase deposition device.

**Table 1. Contact Angle Measurements on Glass Slides**

| substrate | liquid | treatment | angle (deg) | standard deviation | number of measures |
|---|---|---|---|---|---|
| glass slides | pure water | none | 34.2 | 7.0 | 27 |
| | | sulfochromic at 70–80 °C | <15 | | |
| | | blank | 40.6 | 1.7 | 16 |
| | | silanization | 71.5 | 1.8 | 27 |

**Table 2. Surface Roughness Parameter Evolution**

| substrate | roughness (rms) (nm) |
|---|---|
| nontreated glass slide | 0.3 |
| silanized glass slide | 0.7 |
| silanized glass slide + antibodies | >5 |

found to resist acid washing. During such treatments, a partial reorganization of the antibody layer was observed, inducing an increase in antibody activity (*10*).

The efficiency of such a silanization process resulting in a high rate of immobilized antibodies on the silica surfaces has been demonstrated by using glass slides which allow various methods of physicochemical characterization.

Subsequently, antibodies anchored on floating silica hollow microbeads were proved to be able to recognize CD45 rat cells.

The originality of this study is that such hollow, low-density silica microbeads have never been used before. This solid support was chosen because it is inexpensive and could also be used in ultrasound applications. Cavitation could be used for the destruction of cells in vitro (*11*): gas bubbles in the suspension could collapse and damage cell membranes causing their death. Thus, acoustic cavitation could be used as an alternative medical therapy for cancer treatment. F. Prat et al. (*12*) obtained subsequent hepatic lesions in vivo in the rabbit by acoustic cavitation, coupling the injection of artificial bubbles in the liver with ultrasonic treatment. It is worth noting that pressures generated during the collapse could attain tens of thousands of bars (*13*).

## EXPERIMENTAL SECTION

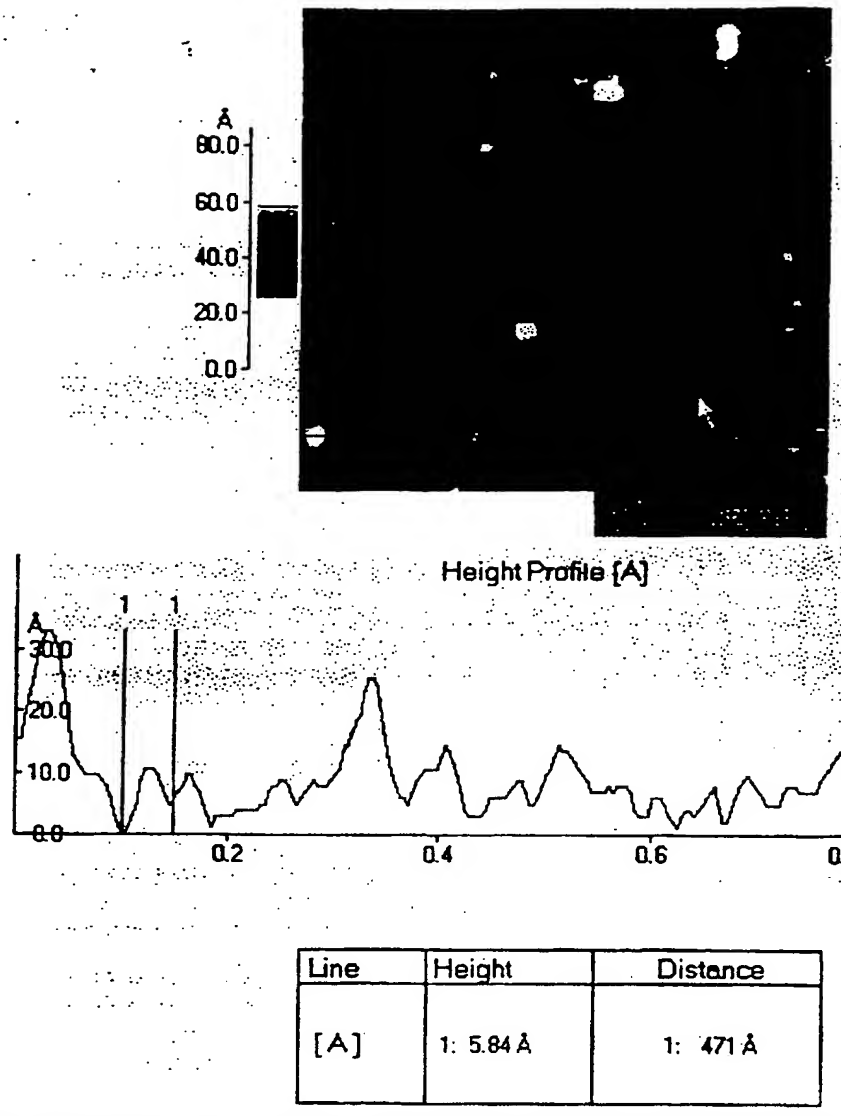
**Reagents.** 3-Cyanopropyl dimethyl chlorosilane was purchased from Aldrich and was used as received. According to the suppliers, its purity was 95%, the remaining 5% being mainly composed of disilane formed which resulted from the condensation of the molecule and was nonreactive toward the surface. The following commercial analytical grade reagents were used: sulfuric acid and potassium dichromate from Merck, tetrahydrofuran from Sigma, xylene from Carlo Erba, and 2-methylbutane from Aldrich.

Low-density hollow silica microbeads (SDT-60:0.6 g $cm^{-3}$) were obtained from NewMet.

The sulfochromic solution was prepared by mixing 1 g of potassium dichromate, 5 mL of deionized water, and 80 mL of 96% concentrated sulfuric acid.

Purified sheep anti-mouse monoclonal antibody, purified mouse anti-rat CD45RC (clone OX22) monoclonal antibody, and biotin-conjugated mouse anti-rat CD45RC (clone OX22) monoclonal antibody were obtained from Pharmingen. A FITC-conjugated $F(ab')_2$ fragment of goat anti-mouse antibody was purchased from Dako. Ficoll was obtained from Dutscher.

Phosphate-buffered saline (PBS), bovine serum albumin (BSA), and Tween 20 were from Sigma, carbonate bicarbonate buffer was prepared by mixing $Na_2CO_3$ 0.1 M and $NaHCO_3$ 0.1 M and adjusting the pH to 9.4. Barbital (Veronal) buffer was prepared by dissolving 4.1 g of sodic veronal and 0.8 g of veronal in 1 L of distilled






| Line | Height | Distance |
|---|---|---|
| [A] | 1: 5.84 Å | 1: 471 Å |

**Figure 5.** AFM image of silanized surface and profile corresponding to the line shown in the image. The height and the distance are measured between lines 1.

water and adjusting the pH to 8.6. Citrate buffer was prepared by mixing 1.78 g of $Na_2HPO_4$, 0.7 g of citric acid, and 134 mL of distilled water.

**Sample Surface Preparation.** Silica surface cleaning and hydroxylation are important in the grafting process. Glass slides were first degreased under ultrasonic stirring with acetone. Due to the difficulties of handling them, silica microbeads were not degreased before the hydroxylation treatment. This first step is not critical, as grease residues should be completely eliminated in the following which involves strong oxidizing reagents.

Glass slides and floating silica microbeads were treated with sulfochromic solution for 30 min at 70–80 °C to remove any coatings or contaminates and to create hydroxyl reactive functions at the surface. Glass slides were washed with deionized water and dried by a pure nitrogen stream. The washing procedure used for microbeads is more critical since they are far more difficult to handle than glass slides. After the sulfochromic treatment, the microbeads were allowed to float to the top of the suspension while the underlying fluid was drawn off. Then deionized water was added to the microbeads which were once again allowed to float. This process was repeated until the sulfochromic solution was completely eliminated. Then, the microbeads were partially dried at 100 °C in air.

**Silane Activation (Figure 3).** After the cleaning process, the glass slides and microbeads were dried at 140 °C under vacuum (0.1 Pa) for 2 h in order to remove the physisorbed water (Figure 4).

For the glass slides, pure silane was introduced under nitrogen atmosphere whereas the microbeads were first placed in a solution of $5 \times 10^{-2}$ mol of silane in 2-methyl butane, to ensure that they would be well wetted. For the latter, the solvent was removed in the following step under vacuum.

The condensation reaction on the silica sample was performed in sealed vessels under vacuum for 48 h at 70 °C.

The excess silane was eliminated by washing with THF, xylene, and deionized water. The glass slides were blown dry with dry nitrogen, whereas the microbeads, were allowed to dry in air at ambient temperature. Although the cleaning and the treating processes are slightly different for each substrate, the surface reactivities to silane reagents are assumed to be quite similar.

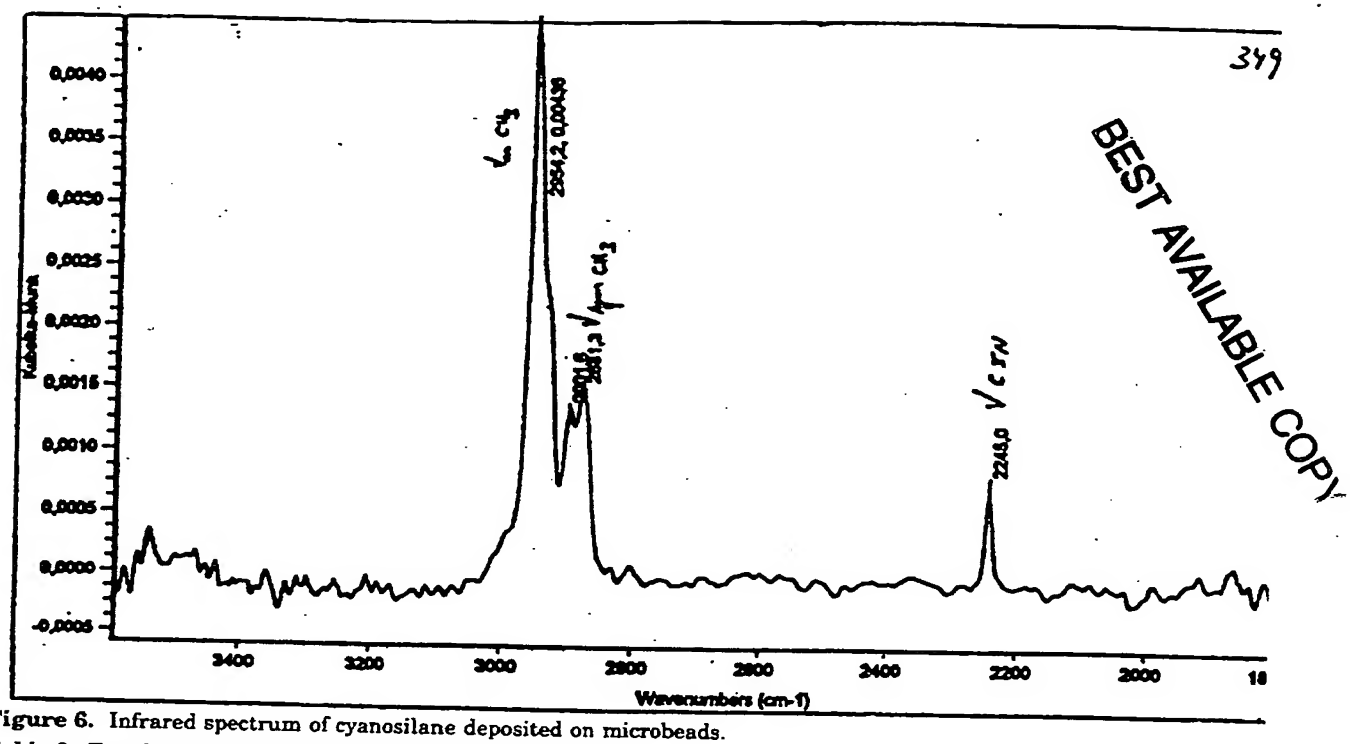



**Figure 6.** Infrared spectrum of cyanosilane deposited on microbeads.

**Table 3. Results of the Cell Binding Assays**

| Substrate and fixation mode | Results | Schematic representation of molecular assemblies |
|---|---|---|
| (1) silane-activated microbeads with CD45 Ab | +/- | |
| (2) non-silanized microbeads sensitized cells | - | |
| (3) silane-activated microbeads with SAM Ab non sensitized cells | - | |
| (4) silane-activated microbeads without SAM Ab sensitized cells | +/- | |
| (5) silane-activated microbeads with SAM Ab sensitized cells | + | |

Silica microbead

Rat cells

SAM Ab

CD45 Ab

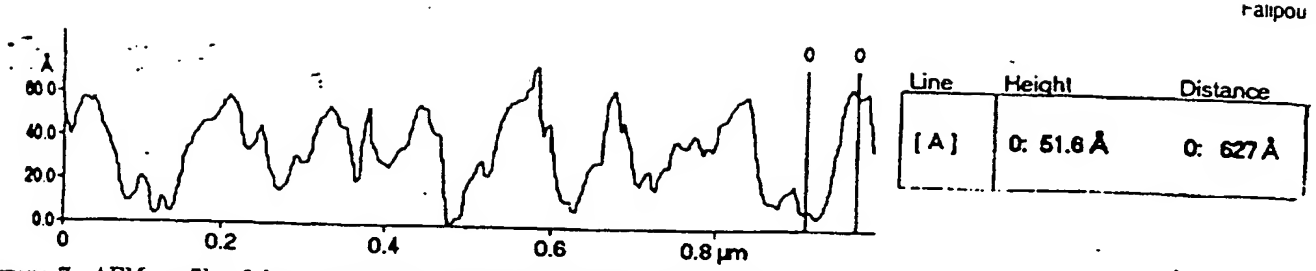



Figure 7. AFM profile of the surface after antibody grafting. The height and the distance are measured between lines 0.

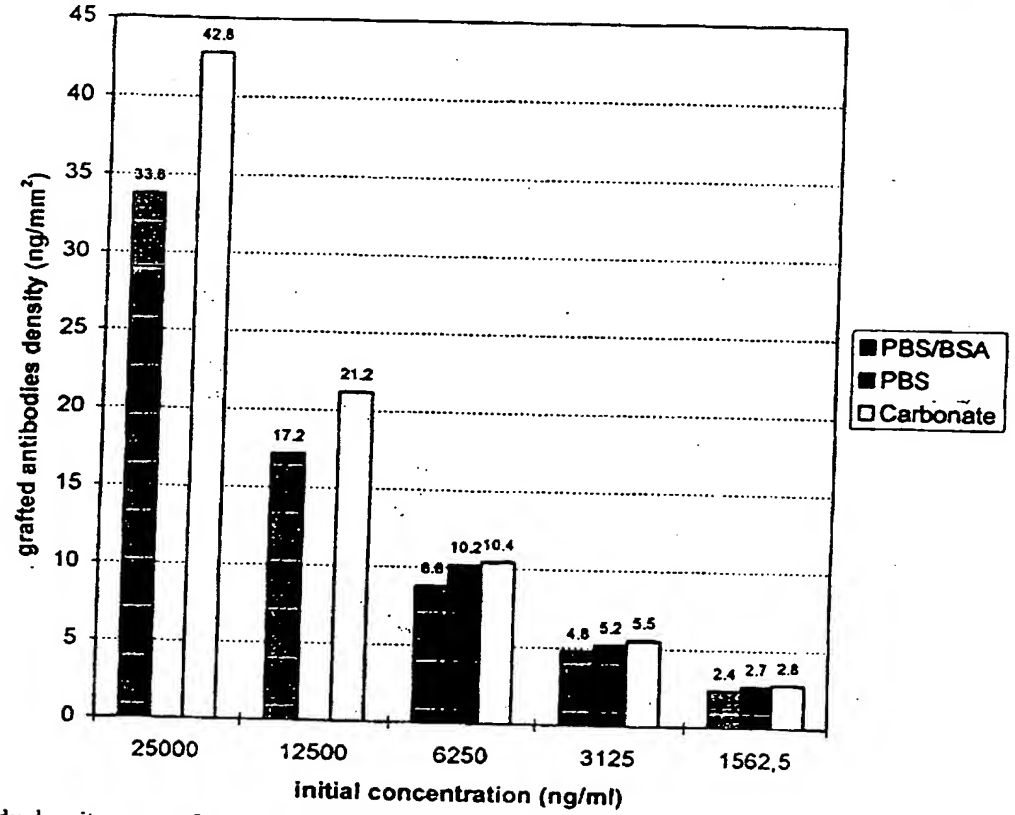



Figure 8. Antibody density per surface unit on glass slide versus the initial concentration for different buffers.

Of course, the possibility that solvent may penetrate the cyanosilane molecules of the microbeads is not excluded.

**Contact Angle Measurements on Planar Substrates.** As the silanized surfaces are more hydrophobic compared to the hydroxylated ones, the efficiency of the silanization process can be controlled by measuring the contact angles.

Contact angles were measured by the sessile drop method on a Digidrop instrument. (GBX Scientific Instruments).

Measurements were made on drops of pure water deposited at 22 °C under an atmosphere saturated with humidity to obtain reproducible conditions.

A video camera equipped with an image analyzer visualized the shape of the drop and gave the contact angle between water drop and silica surface.

The reported contact angles are the average of at least 15 drops for each surface treatment. The macroscopic homogeneity of the surface structure can be estimated by its standard deviation. These measurements are used to control each sample at each step of the silanization process by reference to the values obtained for at least 3 samples and 15 drops of distilled water.

**Atomic Force Microscopy.** Atomic force microscopy (AFM) images were obtained in air using a Park Scientific Instrument (Autoprobe). V-shaped cantilevers (PSI) with a normal spring constant of 0.05 N $m^{-1}$ and a pyramidal $Si_3N_4$ tip were used in this study. Either 100 × 100 $\mu m^2$ or 5 × 5 $\mu m^2$ scanners were used. Images were flattened with only a second order correction. Images were taken in the contact or tapping modes.

Surface evolution was tracked using root-mean-squared surface roughness (rms), measured for each step of the silanization and antibody fixation processes.

**Infrared Spectroscopy on Microbeads.** Methods previously described do not allow the characterization of microspherical substrates. Infrared measurements were made by the Service Central d'Analyses of the CNRS on a Nicolet 20SXC instrument (diffuse reflection mode). Results were given after accumulations of 2048 scans.

**Immobilization of Immunological Species on Silanized Substrates and Analysis of Ab Content by Competitive ELISA Assay: Use of Glass Slides for Technical Purposes.** Mouse anti-rat CD45RC antibody was placed on silanized slides of defined surfaces at concentrations ranging from a few nanograms per milliliter to several micrograms per milliliter. Antibodies were allowed to adsorb for 1 h at 37 °C and 1 night at 4 °C in a wet atmosphere. Various buffers for dilution and washing were studied: phosphate-buffered solution, pH 7.4 (PBS),

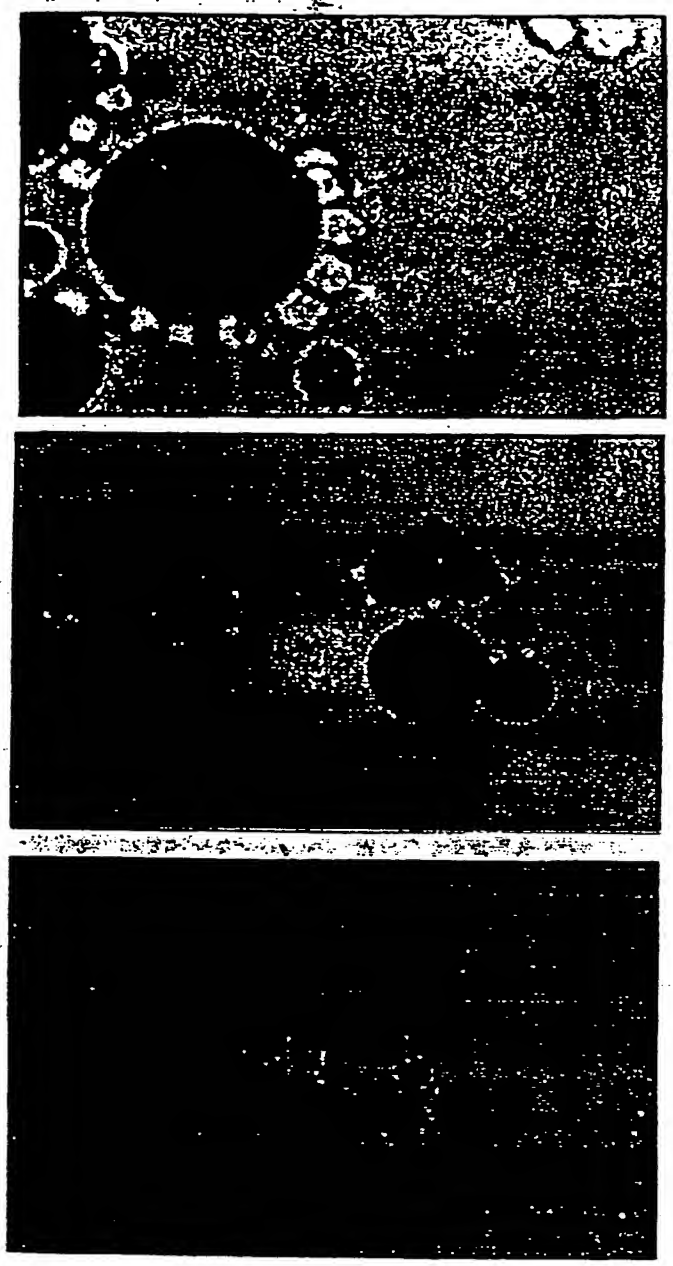


**Figure 9.** Photographs of cells around silica microbeads.

phosphate-buffered solution/bovine serum albumine at pH 7.4 (PBS/BSA) and carbonate/bicarbonate at pH 9.4.

Concentrations of less than 5 $\mu$g/mL could not be obtained by direct enzymatic methods of quantification (for example with a goat anti-mouse horseradish peroxidase monoclonal). Thus, a competitive technique was used in association with the amplified visualization of antibody fixation (an enzymatic reaction with biotinylated mouse antibody and a streptavidin/enzyme complex for binding).

First the optimum optical density curve for biotinylated anti CD45 dilutions was defined. The dilution which gave 50% of the maximal OD was retained.

Second, optical density was tested when nonbiotinylated anti-CD45 antibody dilutions were added to the previously defined biotinylated anti-CD45 dilution.

Finally, the same competition analysis was carried out for the glass slides: the glass slides and 400 $\mu$L Mouse anti-CD45 in concentrations ranging from a few nanograms per milliliter to several micrograms per milliliter were incubated with constant agitation for 1 h at 37 °C and 1 night at 4 °C. The quantity of nonbound antibodies remaining in the liquid was measured by competing with biotin-conjugated antibody at the previously determined concentration, and the results obtained by comparison with the curve defined at the second step were read at 492 and 620 nm. The difference between the total amount used for incubation and the residual one after incubation represented the amount of absorbed antibodies per surface unit.

**Preparation of Rat Lymphocytes for Cell-Immobilization Experiments.** Fresh rat blood (approximately 15 mL) was collected and heparinized. The blood was diluted by half, and 15 mL of diluted blood was added to 50 mL centrifuge tubes containing 15 mL of Ficolite. The tubes were then centrifuged at 1000*g* for 13 min at ambient temperature to obtain a cell density gradient. The resulting layer of peripheral blood lymphocytes, which possess CD45 antigen on their surface, was delicately removed from each tube and pooled.

Cells were washed in PBS containing albumin (PBS/BSA 1%) and spun down 3 times for 13 min at 500*g* and low temperature. A batch of four tubes, each containing $1 \times 10^7$ cells in 50 $\mu$L of PBS/BSA, was prepared, and 100 $\mu$L of a 5 $\mu$g/mL dilution of anti-CD45 antibody was added to each tube before incubation for 30−60 min at 4 °C.

After washing in PBS/BSA, cells were counted with a hemocytometer for viability.

**Immobilization of Cells onto Silica Microbeads.** The experiments and control were carried out using silanized or nonsilanized substrates and different fixation modes were tested using monoclonal mouse anti rat antibodies, which recognize CD45 molecules expressed on rat lymphocytes. Another biochemical link was tested with the insertion of an antibody between the complex cells/CD45 and the substrate SAM Ab.

In all cases, cells were added to the microbeads in a ratio higher than $5 \times 10^5$ cells/mg of microbeads.

The volume was 500 $\mu$L to 1 mL, and the microbeads and cells were incubated for 2 h at 4 °C with agitation. After incubation, centrifuging at 450−500*g* allowed microbeads to float to the top of the suspension whereas nonfixed cells fell to the bottom. Then, the effluent was drawn off. Floating microbeads were observed under the microscope and photographs of the cell were taken.

## RESULTS AND DISCUSSION

**Silanization Process.** Considering the geometry of the silica beads, classical methods of characterization of the silanization cannot be applied. Of these methods, the contact angle measurements offer the most significant wettability test when pure fluids and smooth surface are used. So, to evaluate the wettability after silanization on flat sample, glass slides were used.

Such silica-based samples should behave similarly to microbead ones.

For silane-treated and untreated glass slides, Table 1 summarizes contact angle results at equilibrium.

When no treatment was applied, the value obtained showed great standard deviation ($\theta = 34.2 \pm 7°$), reflecting the heterogeneity of the surface.

The surface of glass slides, when treated by hot sulfochromic solution, is almost totally wetted by water ($\theta < 15°$) demonstrating the efficiency of such a cleaning procedure. Surface hydrophilicity is due to the presence

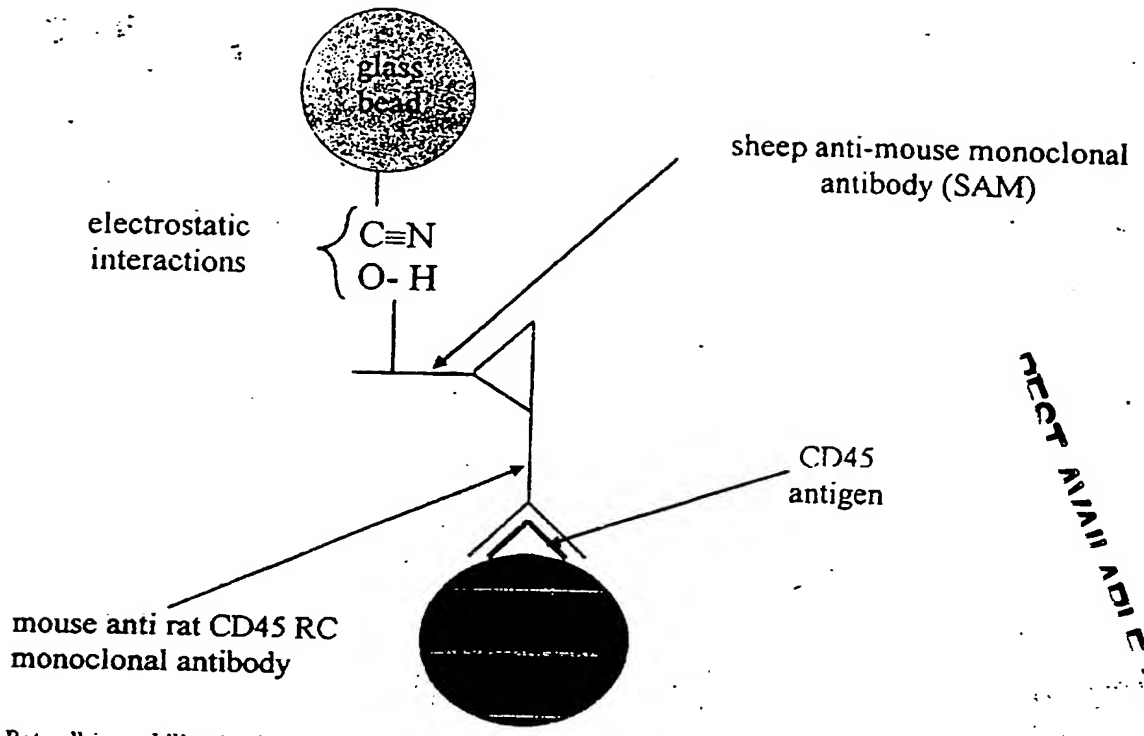



**Figure 10.** Rat cell immobilization by silane-activated glass beads with SAM Ab.

of a great number of hydroxyl groups on the surface which are required for the silanization process (Figure 4). Great care must be taken with cleaned samples, as organic molecules can readily be adsorbed on these reactive surfaces, inducing an increase of the contact angle within a few minutes. Because of this, as soon as the treatment is finished, the samples are placed under vacuum.

After silanization, contact angles increase up to around 71.5 ± 1.8° proving the presence of silane. This surface treatment induces a more homogeneous surface (low standard deviation).

To be sure that no contamination takes place, a sample treated by this process but without the silanization step was tested. In this condition, the value of the contact angle increased up to 40.6°: lower than the value obtained with the silane, but higher than that obtained after sulfochromic treatment.

AFM technique was used to characterize the molecular organization of silane on glass slides. Substrate modifications after reaction were checked by measuring surface roughness variations.

Table 2 shows the evolution of this surface roughness parameter (rms).

The roughness of the silanized surface was found to be higher than the untreated one (rms = 0.7 nm compared to 0.3 nm), values which could be compared to those obtained by Perrin et al. (*14*) who used the same chain length for the silane reagent.

Figure 5 obtained in tapping mode (1 $\mu$m × 1 $\mu$m) shows that the silane layer was dense and relatively homogeneous, except for a small number of pinhole defects. Thanks to these defects, the thickness of one layer was evaluated at 0.58 nm, as shown in Figure 5. This figure also shows a thicker area, proving that in some cases aggregates can be formed. It is noteworthy that by using silane molecules with long alkyl chains [Si–$(CH_2)_{17}CH_3$, for example], a monolayer with almost no defects was observed (*15*). Such aggregates are known (*16*) to be induced by nonuniform reactions on the surface which lead to the formation of at least two different distributions of attached ligands: the expected siloxane bonds and hydrogen bonds between cyano groups and free surface silanols. Although nonuniform reactions take place, the architecture of the film obtained thus appears quite similar for various tested samples. In addition, these interactions are strong enough to resist several washing processes.

The presence of silane on treated microbeads was confirmed by the diffuse reflection infrared spectroscopy method.

Figure 6 shows the presence of nitrile and alkyl functions at the surface of the microbeads, whereas no peaks were obtained with untreated microbeads.

According to the infrared analysis conditions used and taking into account the sensitivity of the apparatus, if only one monolayer was present on the surface, no peak would be visible. In our case, the presence of nitrile and alkyl peaks confirmed that the silanization process had occurred, forming a few aggregates.

In this context, silanization effects would appear to similar for both substrates.

**Immobilization of Immunological Species.** Figure 7 shows an AFM profile taken in tapping mode in air from antibodies deposited onto cyanosilane. The profile showed aggregates which were about 5 nm high. These aggregates suggest that lateral interactions occur between the adjacent IgG molecules. It was not surprising to find such lateral interactions, as this protein has some self-aggregating properties.

The height of these molecules roughly matched the known size of one IgG molecule (5 × 15 nm) (*4*) set horizontally confirming the orientation of the antibodies and thus the nature of the link (electrostatic interactions between OH groups of the glycosylated regions on the Fc fragment of antibodies and CN functions of the silane).

**Optimization of Antibody Fixation: ELISA Assay.** The ELISA assay was done with mouse anti-rat CD45RC

in concentrations ranging from a few nanograms per milliliters to several micrograms per milliliter in different buffers: PBS, PBS/BSA (1%), and carbonate/bicarbonate. This assay was used to determine the optimum mouse anti-rat CD45RC level coupled to well-defined surfaces of glass slides activated with the cyanosilane by varying the antibody concentration and the incubation buffer.

The antibody surface density was given as a function of initial antibody concentration in solution for three buffers.

Results are shown in Figure 8. Concerning the nonspecific binding, measurements carried out on unsilanized slides lead to an adsorption rate which never exceeds 3%.

These results showed the validity of the cyanosilane coupling agent for the immobilization of antibodies onto glass or silica surfaces.

The most suitable buffer was found to be the carbonate buffer with a pH of 9.4.

**Cell Binding.** Table 3 compares cell immobilization behavior in various fixation modes. The fixation control was carried out by a camera coupled to an optical microscope, and results were expressed as + for subsequent immobilization (a mean number of at least one cell fixed to bead after centrifuging at 450−500*g*), ± for weak immobilization (some beads fix no cells), and − for no immobilization of cells on the substrate.

This table shows that only activated microbeads with SAM Ab incubated with sensitized cells subsequently immobilize cells (in the table, the fifth substrate and fixation mode). In this case, microbeads grafted with SAM Ab, which acts as a spacer, allow cells sensitized with CD45 Ab to approach the microbeads better, which is in good agreement with the literature (*17*). Figure 9 shows photographs of cell aggregation around microbeads. The architecture of the biochemical link between cells and microbeads is given in Figure 10. Moreover, by fixing SAM Ab, which is able to recognize mouse species on lymphocytes, to substrates, unexpected antibody fixation to or removal from substrates could be minimized.

The experimental procedure was validated by producing blanks to confirm that no fixation occurs when microbeads were not silanized and also that SAM Ab does not recognize CD45 molecules expressed on lymphocytes.

## CONCLUSION

By applying a specific functionalization process, activated hollow silica microbeads were shown to be able to fix rat cells strongly and selectively. Efficient fixation of antibodies was obtained with a reproducible silane layer, through electrostatic interactions between the OH groups of the glycosylated regions on the Fc fragments and CN groups of the silane. The recognizing capabilities of the Fab groups are preserved when this type of fixation method is used. A further step could be the destruction of the cells as the microbeads could be destroyed under ultrasonic power. Such a process could be used successfully to separate different strains of cells.

## LITERATURE CITED

(1) Nelson, J. M., and Griffin, E. G. (1916) *J. Am. Chem. Soc. 38*, 1109.

(2) Maupas, H. (1995) Thesis of Ecole Centrale Lyon.

(3) Maupas, H., Saby, C., Martelet, C., Jaffrezic-Renault, N., Soldatkin, A. P., Charles M−H., Delair, T., and Mandrand, B. (1996) *J. Electroanal. Chem. 406*, 53.

(4) Harlow, E., and Lane D. (1991) *Anticorps: un manuel de laboratoire*, Editions Pradel, Paris.

(5) Karraasch, S., Dolder, M., Schabert, F., Ramsden, J., and Engel A. (1993) *Biophys. J. 65*, 2437.

(6) Browning-Kelley, M. E., Wadu-Mesthrige, K., Hari, V., and Liu, G. Y. (1997) *Langmuir 13*, 343.

(7) Taylor, R. F. (1991) Fundamentals and Applications. *Protein immobilization*, Marcel Dekker Inc.

(8) Plueddemann, E. P. (1982) *Silane Coupling Agents*, Plenum Press, New York.

(9) Saby, C., Jaffrezic-Renault, N., Martelet, C., Colin, B., Charles, M.-H, Delair, T., and Mandrand D. (1993) *Sensors Actuators B, 15−16*, 458.

(10) Billard, V., Martelet, C., Binder, P., and Therasse, J. (1991) *Anal. Chim. Acta 249*, 367.

(11) Prat, F., Chapelon, J. Y., Chauffert, B., Ponchon, T., and Cathignol, D. (1991) *Cancer Res. 51* (11), 3024.

(12) Prat, F., Ponchon, T., Berger, J. Y., Chapelon, J. Y., Gagnon, P., and Cathignol, D. (1991) *Gastroenterology 100* (5), 1345.

(13) Church, C. (1989) *J. Acoust. Soc. Am. 86* (1), 215.

(14) Perrin, A., Lanet, V., and Theretz, A. (1997) *Langmuir 13*, 2557.

(15) Duchet, J. *Système modèle Polyéthylène/verre: rôle de chaînes connectrices greffées sur l'adhésion*, Thesis, Université Claude Bernard Lyon 1.

(16) Suffilk, B. R., and Gilpin, R. K. (1986) *Anal. Chim. Acta 181*, 259.

(17) *DYNAL*, Technical Handbook, second ed.

BC9800421